

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. § 371

418 Rec'd PCT/PTO 0.1 APR 1999  
Patent and Trademark Office  
Docket No. 402162000200

09/269874

U.S. APPLICATION NO. (If known, see 37 C.F.R. § 1.5): Not Yet Assigned

INTERNATIONAL APPLICATION NO.

PCT/EP97/05441

INTERNATIONAL FILING DATE

October 2, 1997

PRIORITY DATE CLAIMED

October 2, 1996

TITLE OF INVENTION: RECOMBINANT PROCESS FOR PREPARING A COMPLETE MALARIA ANTIGEN, gp190/MSP1

APPLICANT(S) FOR DO/EO/US: Hermann BUJARD, Ralf TOLLE, and Weiqing PAN

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. § 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date (copy enclosed).
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9. ☒ An unsigned declaration of the inventor(s) (35 U.S.C. § 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§ 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment (7 pages).
 ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Check in the amount of \$1,556.00; Return Receipt Postcard


CERTIFICATE OF MAILING BY "EXPRESS MAIL"

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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

*Tiffany Dolmseth*  
Tiffany Dolmseth

U.S. APPLICATION NO. (If known, see 37 C.F.R. § 1.5)		INTERNATIONAL APPLICATION NO. PCT/EP97/05441		DOCKET NUMBER: 99971-0000029	
17. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 C.F.R. §§ 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO ..... \$840.00 International preliminary examination fee paid to (USPTO (37 C.F.R. § 1.482))..... \$670.00 No international preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) but international search fee paid to USPTO (37 C.F.R. § 1.445(a)(2))..... \$760.00 Neither international preliminary examination fee (37 C.F.R. § 1.482) nor international search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO..... \$970.00 International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... \$96.00				<b>CALCULATIONS PTO USE ONLY</b>	
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$970.00	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)).				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	41 - 20 =	21	x \$18.00	\$378.00	
	4 - 3 =	1	x \$78.00	\$ 78.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$ 0	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$1556.00	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§ 1.9, 1.27, 1.28)				\$0	
<b>SUBTOTAL =</b>				\$1556.00	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)). +				\$0	
<b>TOTAL NATIONAL FEE =</b>				\$1556.00	
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). <b>\$40.00 per property +</b>				\$	
<b>TOTAL FEES ENCLOSED =</b>				\$1556.00	
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				<b>charged:</b>	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$1,556.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my <u>Deposit Account No. 03-1952</u> in the amount of \$0 to cover the above fees. c. <input checked="" type="checkbox"/> The Assistant Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to <u>Deposit Account No. 03-1952 (Our Ref. 402162000200)</u> .					
<b>NOTE: Where an appropriate time limit under 37 C.F.R. § 1.494 or 1.495 has not been met, a petition to revive          (37 C.F.R. § 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO: <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 45%;">           Paula A. Borden            Morrison &amp; Foerster LLP            755 Page Mill Road            Palo Alto, California 94304-1018         </div> <div style="width: 45%; text-align: center;">             Paula A. Borden            Registration No. 42,344         </div> </div>					



## I. AMENDMENT

### In the specification

At page 1, after the title, please insert the following sentence.

-- This application is a 35 U.S.C. §371 filing of International Patent Application No. PCT/EP97/05441, filed October 2, 1997. This application claims priority benefit of German Patent Application No. DE 19640817.2, filed October 2, 1996.

### In the Claims:

Please cancel claims 1-41, without prejudice or disclaimer. Please add new claims 42-82, shown below.

- 42. A method for producing a complete gp190/MSP1 protein of a *Plasmodium*, comprising expressing a nucleotide sequence encoding the complete gp190/MSP1 protein in an expression vector, wherein the AT content of the expressed nucleotide sequence encoding the complete gp190/MSP1 protein is less than the AT content of a naturally occurring nucleotide sequence encoding a gp190/MSP1 protein.
43. The method of claim 42, wherein the *Plasmodium* is a strain of *Plasmodium falciparum*.
44. The method of claim 43, wherein the strain of *Plasmodium falciparum* is *P. falciparum* strain FCB-1.
45. The method of claim 42, wherein the AT content is reduced from about 74% to 55%.
46. The method of claim 42, wherein the nucleotide sequence encoding the complete gp190/MSP1 is denoted gp190s in Figure 3C.
47. The method of claim 42, wherein the nucleotide sequence encoding a gp190/MSP1 protein further comprises a nucleotide sequence encoding an attachment signal.

48. The method of claim 42, wherein the nucleotide sequence encoding a gp190/MSP1 protein further comprises a nucleotide sequence encoding a signal peptide.
49. The method of claim 42, wherein nucleotide sequence encodes a gp190/MSP1 having the amino acid sequence selected from the group consisting of amino acids 1-1639, 1-1621, and 20-1621, depicted in Figure 3C.
50. The method of claim 1, wherein the nucleotide sequence encoding the complete gp190/MSP1 protein is produced according to a method comprising:
- (a) designing a nucleotide sequence encoding a gp190/MSP1 from a *Plasmodium* to contain codon frequencies common in the human genome, while maintaining the encoded gp190/MSP1 protein;
  - (b) dividing the sequence of step (a) into individual overlapping regions;
  - (c) synthesizing desoxynucleotides each extending the whole length of each individual region of step (b);
  - (d) cloning the individual region coding sequences; and
  - (e) fusing the regions to produce a sequence encoding gp190/MSP1.
51. The method of claim 50, wherein the nucleotide sequence being designed encodes a gp190/MSP1 protein from *Plasmodium falciparum* FCB-1, and the overlapping regions of step (b) encode p83, p31, p36, p30, and p19, and wherein the method further comprises a step (c') which is performed after step (c) and comprises synthesizing coding regions encoding gp19, gp30, p36 and p31 by polymerase chain reaction and synthesizing a coding region encoding p83 by fusion of two sequences comprising approximately 1200 bp.

52. The method of claim 50, wherein the desoxyoligonucleotides synthesized in step (c) are on average 120 nucleotides long, and wherein the overlap between individual regions is about 20 bases.
53. The method of claim 42, wherein the expression vector is selected from the group consisting of dPS56RBSII, pBi-5, and ppTMCS.
54. The method of claim 42, wherein the nucleotide sequence is expressed in an *Escherichia coli* (*E. coli*) strain.
55. The method of claim 54, wherein the *E. coli* strain is DH5alphaZ1.
56. The method of claim 42, wherein the nucleotide sequence is expressed in an expression system selected from the group consisting of HeLa cells and CHO cells.
57. The method of claim 42, wherein the nucleotide sequence is expressed in an expression system selected from the group consisting of *Toxoplasma gondii* and *Leishmania*.
58. A nucleotide sequence encoding a complete gp190/MSP1 protein of a *Plasmodium*, wherein the AT content of the nucleotide sequence encoding the complete gp190/MSP1 protein is less than the AT content of a naturally occurring nucleotide sequence encoding a gp190/MSP1 protein.
59. The nucleotide sequence of claim 58, further comprising a nucleotide sequence encoding an attachment signal.
60. The nucleotide sequence of claim 58, further comprising nucleotide sequences encoding a signal peptide.
61. The nucleotide sequence of claim 58, wherein the sequence further comprises a sequence encoding an N-terminal extension of about 11 amino acids, of which six are histidine.
62. The nucleotide sequence of claim 58, wherein the sequence lacks sequences that function as splice donor and splice acceptor signals.

63. The nucleotide sequence of claim 58, wherein the sequence lacks a region comprising GC-rich sequences.
64. The nucleotide sequence of claim 58, wherein the sequence lacks recognition sequences for restriction enzymes which recognize sequences of six or more base pairs.
65. The nucleotide sequence of claim 58, wherein the nucleotide sequence comprises one or more cleavage sites for restriction endonucleases, and wherein each of said cleavage sites occurs no more than once in said nucleotide sequence.
66. The nucleotide sequence of claim 58, further comprising cleavage sites for endonucleases at the 5' and 3' ends of the nucleotide sequence, said endonuclease cleavage sites being absent from the remainder of the nucleotide sequence and from an expression or cloning vector containing said nucleotide sequence.
67. The nucleotide sequence of claim 58, wherein the nucleotide sequence encoding the complete gp190/MSP1 consists of a sequence denoted gp190s in Figure 3C.
68. The nucleotide sequence of claim 58, wherein the AT content is reduced from about 74% to about 55%, compared to the AT content of the naturally occurring nucleotide sequence.
69. The nucleotide sequence of claim 58, wherein nucleotide sequence encodes a gp190/MSP1 having the amino acid sequence selected from the group consisting of amino acids 1-1639, 1-1621, and 20-1621 of the sequence denoted gp190s in Figure 3C.
70. A vector comprising the nucleotide sequence of claim 58.
71. The vector of claim 70, wherein the vector is an expression vector.
72. The vector of claim 71, wherein the expression vector is selected from the group consisting of dPS56RBSII, pBi-5, and ppTMCS.
73. A host organism comprising the nucleotide sequence of claim 58.

74. The host organism of claim 73, wherein the host organism is an *Escherichi coli* (*E.coli*) strain.
75. The host organism of claim 74, wherein the *E. coli* strain is DH5alphaZ1.
76. The host organism of claim 73, wherein the host organism is a cell selected from the group consisting of a HeLa cell and a CHO cell.
77. The host organism of claim 76, wherein the cell constitutively synthesizes a tetracycline transactivator.
78. The host organism of claim 73, which is selected from the group consisting of *Toxoplasma gondii*, *Leishmania*, baculovirus, adenovirus, and yeast.
79. A method of delaying development of malaria parasite *Plasmodium* parasitemia in an animal, comprising administering gp190/MSP1 to the animal.
80. A composition for use as a vaccine, said composition comprising a nucleotide sequence according to claim 58.
81. A composition comprising a host organism according to claim 73.
82. A method for stabilizing a gene sequence, comprising reducing the AT content of the sequence, compared to the AT content of the naturally occurring gene sequence.

## II. REMARKS

The PCT application contained claims 1-41. By this Preliminary Amendment, Applicants have cancelled original claims 1-41, without prejudice or disclaimer, and have added



new claims 42-82. Claims 42-82 are now pending in this application. Support for the new claims is found in the original claims and throughout the specification as filed.

### III. SUMMARY

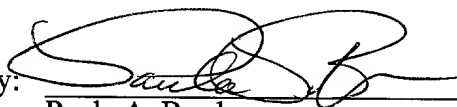
Prior to examination, please enter the amendments submitted herein.

If a telephone call would further prosecution of this case, the Examiner is invited to call the undersigned at (650) 813-5776. In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 402162000200. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: April 1, 1999

By:



Paula A. Borden  
Registration No. 42,344

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Applicant/Patentee: **Hermann Bujard, Ralf Tolle, Weiqing Pan** Docket No.: **402162000200**  
Serial No./Patent No.: **Not Yet Assigned (National Phase Filing of PCT/EP97/05441)**  
Filed on/Issued: **April 1, 1999**  
For: **RECOMBINANT PROCESS FOR PREPARING A COMPLETE**  
**MALARIA ANTIGEN, gp190/MSP1**

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS**  
**37 C.F.R. §§ 1.9(f) AND 1.27(b) - INDEPENDENT INVENTOR**

As a below-named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 C.F.R. § 1.9(c) for purposes of paying reduced fees to the Patent and Trademark Office described in:

☒ **the National Phase Filing of PCT/EP97/05441.**

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

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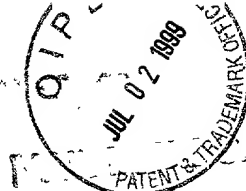
NAME	ADDRESS	TYPE
		<input type="checkbox"/> Individual <input type="checkbox"/> Small Business Concern <input type="checkbox"/> Nonprofit Organization

**NOTE:** Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Inventor	Signature of Inventor	Date
<b>Hermann BUJARD</b>		<b>May 12, 1999</b>



Applicant/Patentee:	Hermann Bujard, Ralf Tolle, Weiqing Pan	Docket No.:	402162000200
Serial No./Patent No.:	Not Yet Assigned (National Phase Filing of PCT/EP97/05441)		
Filed on/Issued:	April 1, 1999		
For:	RECOMBINANT PROCESS FOR PREPARING A COMPLETE MALARIA ANTIGEN, gp190/MSP1		

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
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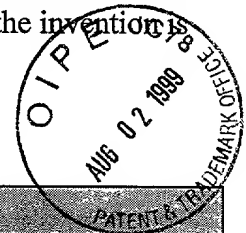
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I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

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- ☐ no such person, concern, or organization.  
☒ each such person, concern or organization is listed below.



NAME	ADDRESS	TYPE
Hermann Bujard	Remlerstrasse 9 D-69120 Heidelberg Germany	<input checked="" type="checkbox"/> Individual <input type="checkbox"/> Small Business Concern <input type="checkbox"/> Nonprofit Organization

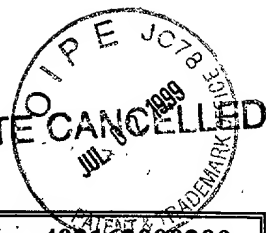
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Inventor	Signature of Inventor	Date
Ralf TOLLE		20.5.99

MAIL DATE CANCELLED



Applicant/Patentee: **Hermann Bujard, Ralf Tolle, Weiqing Pan** Docket No.: **402162000200**  
 Serial No./Patent No.: **Not Yet Assigned (National Phase Filing of PCT/EP97/05441)**  
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 For: **RECOMBINANT PROCESS FOR PREPARING A COMPLETE MALARIA ANTIGEN, gp190/MSP1**

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
 37 C.F.R. §§ 1.9(f) AND 1.27(b) - INDEPENDENT INVENTOR**

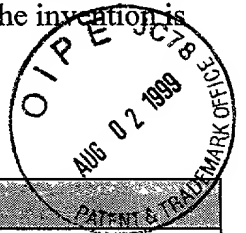
As a below-named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 C.F.R. § 1.9(c) for purposes of paying reduced fees to the Patent and Trademark Office described in:

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I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☐ no such person, concern, or organization.  
☒ **each such person, concern or organization is listed below.**



NAME	ADDRESS	TYPE
<b>Hermann Bujard</b>	<b>Remlerstrasse 9 D-69120 Heidelberg Germany</b>	<input checked="" type="checkbox"/> Individual <input type="checkbox"/> Small Business Concern <input type="checkbox"/> Nonprofit Organization

**NOTE:** Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27)

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Name of Inventor <b>Weiqing PAN</b>	Signature of Inventor <i>W. Pa</i>	Date <i>05.06.1999</i>
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552090-42359250

**Recombinant process for preparing a complete malaria antigen, gp190/MSP1**

The invention concerns a recombinant manufacturing process for the complete malaria antigen gp190/MSP1, as well as separate naturally-occurring domains and parts of the same, by expression of a synthetic DNA sequence. The invention concerns in addition the DNA sequences produced by the process and the host organisms used for the expression of the DNA sequences. In addition the invention concerns the use of the complete malaria antigen as well as parts thereof as a vaccine for immunization against malaria.

Finally the invention under consideration concerns a stabilization process for AT-rich genes, as well as stabilized genes which are characterized by a reduced AT content.

Malaria is one of the most significant infectious diseases in the world. According to WHO reports, in 1990 40% of the world population in 99 countries was exposed to the risk of malaria. At the same time its distribution is enormously on the increase. This may be ascribed above all to intensive development of resistance in the parasites causing malaria, promoted by the recommendation and use as prophylactics of the drugs intended for treatment. Besides the search for new and effective chemotherapeutic agents hope is nowadays directed towards the development of vaccines, since people in areas of the world where malaria is epidemic do manage to develop some kinds of immunity. As well as a natural resistance to malaria, such as that found in heterozygous carriers of the sickle-cell gene and people with thalassaemia and glucose-6-phosphate dehydrogenase deficiency, in the course of malarial infection in humans immune mechanisms can be stimulated which express themselves in a heightened capacity for resistance to the Plasmodia. Consequently the course of the disease in populations exposed to severe epidemics is generally less threatening than in persons exposed to the infection less frequently or for the first time.

The main problem in the development of a vaccine is the identification of an antigen which can induce protective immunity, since there is no easily accessible well-defined animal model available for the four parasites affecting man. The organism causing malaria belongs to the Plasmodium group, of which infection with one of the four parasites Plasmodium vivax,

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Plasmodium ovale, Plasmodium malariae or Plasmodium falciparum results from the bite of Anopheles mosquitoes. Of these parasites Plasmodium falciparum is the most dangerous and the most widely distributed.

The main surface protein of the merozoite, the invasive form of the blood stage of the malaria parasite Plasmodium falciparum and other malaria parasites such as P. vivax, is a 190-220 kD glycoprotein. Late in the development of the parasite this precursor is processed into smaller proteins, which can however be isolated from merozoites as a unitary complex. By means of a glycosylphosphatidyl-inositol bond this complex is coupled to the merozoite membrane. The sequences of the gp190 proteins of various P. falciparum strains fall into two groups, between which intragenic recombination is frequent. In general the protein consists of many highly conserved regions, of a dimorphic zone to which in each case one of two alleles belongs, and of two relatively small oligomorphous blocs in the N-terminal zone (Tanabe, K., Mackay, M., Goman, M. and Scaife, J.G. (1987), Allelic dimorphism in a surface antigen gene of the malaria parasite Plasmodium falciparum. J. Mol. Biol. 195, 273-287, Miller, L.H., Roberts, T., Shahabuddin, M. and McCutchan, T.F. (1993), Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP-1). Mol. Biochem. Parasitol. 59, 1-14).

Already early on gp190/MSP1 was considered as a possible candidate for a vaccine. In the rodent model active protection against infection with rodent parasites was obtained following immunization with the analogous protein. Passive protection could be procured with antibodies directed against this protein (see also Holder, A.A. and Freeman, R.R. (1981), Immunization against blood-stage rodent malaria using purified parasite antigens, Nature 294, 361-364; Marjarian, W.R., Daly, T.M., Weidanz, W.P. and Long, C.A. (1984), Passive immunization against murine malaria with an IgG3 monoclonal antibody, J. Immunol. 132, 3131-3137). The data which ought to support this assumption are nevertheless in details not statistically significant.

There are in addition, a number of monoclonal antibodies which in vitro inhibit the invasion of erythrocytes by P. falciparum and are directed against gp190/MSP1 (Pirson, P.J. and Perkins, M.E. (1985), Characterization with monoclonal antibodies of a surface antigen of Plasmodium

falciparum merozoites. J. Immunol. 134, 1946-1951; Blackman, M.J., Heidrich, H.-G., Donachie, S., McBride, J.S. and Holder, A.A. (1990), A single fragment of a malaria merozoite surface protein remains on the parasite during red-cell invasion and is the target of invasion-inhibiting antibodies. J. Exp. Med. 172, 379-382).

Finally, a series of vaccine studies have been carried out with gp190/MSP1 from *P. falciparum* on primates, particularly on Aotus and Saimiri monkeys (see also Perrin, L.H., Merkli, B., Loche, M., Chizzolini, C., Smart, J. and Richle, R. (1984), Antimalarial immunity in Saimiri monkeys. Immunization with surface components of asexual blood stages, J. Exp. Med. 160, 441-451; Hall, R., Hyde, J.E., Goman, M., Simmons, D.L., Hope, I.A., Mackay, M. and Scaife, J.G. (1984), Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria, Nature 311, 379-382; Siddiqui, W.A., Tam, L.Q., Kramer, K.J., Hui, G.S.N., Case, S.E., Yamaga, K.M., Chang, S.P., Chan, E.B.T. and Kan, S.-C. (1987), Merozoite surface coat precursor protein completely protects Aotus monkeys against Plasmodium falciparum malaria, Proc. Natl. Acad. Sci. USA 84, 3014-3018; Ettlinger, H.M., Caspers, P., Materile, H., Schoenfeld H.-J., Stueber, D. and Takacs, B. (1991), Ability of recombinant or native proteins to protect monkeys against heterologous challenge with Plasmodium falciparum, Inf. Imm. 59, 3498-3503; Holder, A.A., Freeman, R.R. and Nicholls, S.C. (1988), Immunization against Plasmodium falciparum with recombinant polypeptides produced in Escherichia coli, Parasite Immunol. 10, 607-617; Herrera, S., Herrera, M.A., Perlaza, B.L., Burki, Y., Caspers, P., Doebeli, H., Rotmann D. and Certa, U. (1990), Immunization of Aotus monkeys with Plasmodium falciparum blood-stage recombinant proteins, Proc. Natl. Acad. Sci. USA 87, 4017-4021; Herrera, M.A., Rosero, F., Herrera, S., Caspers, P., Rotmann, D., Sinigaglia, F. and Certa, U. (1992), Protection against malaria in Aotus monkeys immunized with a recombinant blood-stage antigen fused to a universal T-cell epitope; correlation of serum gamma interferon levels with protection, Inf. Imm. 60, 154-158; Patarroyo, M.E., Romero, P., Torres, M.L., Clavijo, P., Moreno, A., Martinez A., Rodriguez, R., Guzmán, F. and Cabezas, E. (1987), Induction of protective immunity against experimental infection with malaria using synthetic peptides, Nature 328, 629-632). In these vaccine studies two premises may be distinguished:

- Use of material isolated from parasites, and

- Administration of material procured in heterologous systems of expression.

The latter consists as a rule of relatively small segments of the total protein. Although the results of the inoculations carried out preliminarily on monkeys indicate that gp190/MSP1 could bring about protection, all the experiments carried out on primates have two problems, which place such a conclusion in question:

- (a) they were carried out on too small groups of animals
- (b) they were not repeated.

The results and the conclusions drawn from them are consequently not statistically confirmed. Besides the difficulty of access to suitable monkeys there remains the main basic problem, that it has so far not been possible to manufacture good vaccination material in a suitable quantity.

On the other hand, after the sequencing of the gp190 gene from the K1 and MAD20 strains of *Plasmodium falciparum* overlapping fragments could be expressed in *E. coli*. With this material epidemiological studies in West Africa showed that in the adolescent group a correlation existed between antibody titre against gp190/MSP1 fragments on one hand and protection from parasite infection on the other. In addition the titre also appeared to correlate with the capacity to control the parasitaemia even at a low level (Tolle et al. (1993): A prospective study of the association between the human humoral immune response to *Plasmodium falciparum* blood stage antigen gp190 and control of malarial infections, *Infect. Immun.* 61, 40-47). These results are supplemented by new investigations on Aotus monkeys in the framework of the present invention. Here an enhanced protection against infection with the parasite was attained because protein preparations from *Plasmodium falciparum*, which consisted predominantly of unprocessed gp190/MSP1, had been used as vaccine. The monkeys with the highest antibody titres against gp190/MSP1 were the best protected. These results eventually indicated gp190 as a most promising candidate for a vaccine against tropical malaria.



By some groups of workers the C-terminal domain of gp190 (p19 or p42) is assigned a particular role in the immunity mediated by gp190 (see also Chang, S.P., Case, S.E., Gosnell, W.L., Hashimoto, A., Kramer, K.J., Tam, L.Q., Hashiro, C.Q., Nikaido, C.M., Gibson, H.L., Lee-Ng, C.T., Barr, P.J., Yokota, B.T. and Hui, G.S.N. (1996), A recombinant baculovirus 42-kilodalton C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 protects Aotus monkeys against malaria, *Inf. Imm.* 64, 253-261; Burghaus, P.A., Wellde, B.T., Hall, T., Richards, R.L., Egan, A.F., Riley, E.M., Ripley-Ballou, W. and Holder A.A. (1996), Immunization of Aotus nancymai with recombinant C-terminus of *Plasmodium falciparum* merozoite surface protein 1 in liposomes and alum adjuvant does not induce protection against a challenge infection, *Inf. Imm.*, in press.

Thus far, however, it has also been impossible to exclude other parts of gp190 on a rational basis as irrelevant to a protective immune response. Hence it is as necessary as ever to use the entire gene or the intact gp190 for vaccine investigations. Despite multiple investigations by various work-groups, however, there has not yet been any success in cloning and expressing the entire gp190/MSP1 gene.

Nor has it so far been possible to exclude a priori any part of the gp190 sequence as irrelevant to the protective immune response, so that it is as necessary as ever to use the entire gene or gene product for vaccine investigations. Nevertheless, despite many investigations by a number of working groups there has not yet been any successful cloning of the whole gene for gp190/MSP1.

One object of the present invention has consequently been to make available an adequate quantity of vaccine material in the form of the complete gp190/MSP1. It was a further object of the present invention to provide a process by which this vaccine material could be recovered.

In addition it was another object on the part of the present invention to provide a complete DNA sequence of gp190/MSP1 which could be expressed in a host organism.

Yet another object of the present invention was to provide host organisms containing the complete gp190/MSP1 gene.

Finally, it was also an object of the present invention to provide a stabilization process for AT-rich genes, as well as a stabilized gene suitable for expression characterized in a reduction of the AT content.

These objects are solved by the subject matter outlined in the Claims.

In the following, certain concepts are explained in more detail in order to make clear how they should be understood in this context.

"Recombinant manufacturing process" means that a protein of a DNA sequence is expressed by a suitable host organism in which the DNA sequence has arisen from cloning and fusion of individual DNA fragments.

"Complete gp190/MSP1 protein" here means the entire gp190/MSP1 surface protein isolatable from the above named Plasmodia, especially *Plasmodium falciparum*, representing the main surface protein of the above named parasite as well as the proteins with analogous function from the other *Plasmodium* species such as *P. vivax*. The term therefore comprises in each case the main surface protein of the merozoites of the four malaria parasites named above as dangerous to man. "Complete gp190/MSP1 gene" means the gene coding for this protein. In this context "complete" signifies that the entire amino-acid sequence of the native protein is present or that the gene sequence codes for the entire amino-acid sequence of the native protein. Mutated and/or shortened forms of gp190/MSP1 are however included therewith insofar as they display the same immunization potential (vaccine protection) as the complete gp190/MSP1. Finally the term also includes variants of gp190/MSP1 characterized by containing in one molecule protein fragments of various alleles.

"FCB-1" is a strain of *P. falciparum* such as that described in Heidrich, H.-G., Miettinen-Baumann, A., Eckerskorn, C. and Lottspeich, F. (1989) The N-terminal amino acid sequences of the *Plasmodium falciparum* (FCB1) merozoite surface antigens of 42 and 36 kilodalton, both derived from the 185-195-kilodalton precursor. *Mol. Biochem. Parasitol.* 34, 147-154.

"Attachment signal" here means a protein structure coded for by a DNA sequence at the 3' or 5' end of the gene according to the invention. Attachment signals are structures enabling the attachment of a polypeptide to other structures, such as for example membranes.

"Signal peptide" here signifies a protein structure for which a DNA sequence at the N-terminal end of the gene according to the invention codes. Signal peptides are structures which among other things enable penetration of the polypeptide into membranes.

In the context of the present invention "AT-content" means the percentage amount of adenine-thymine base pairs compared to guanine-cytosine base pairs.

"Cloning" will comprehend here all known state-of-the-art cloning methods which could be applied here, which are nevertheless not all described in detail because they belong among the normal tools of the person skilled in the art.

"Expression in an appropriate expression system" should here include all known state-of-the-art methods of expression in known expression systems which could be applied here, but which are nevertheless not all described in detail because they belong among the normal tools of the person skilled in the art.

It is a primary object in regard to the present invention that a process be provided by which the protein gp190/MSP1 and its gene can be produced in sufficient quantity without excessive cost.

This object is solved by the recombinant manufacturing process set out in Claim 1, by which a complete gp190/MSP1 gene and the protein coded by it are obtainable in sufficient quantities.

For the first time it is possible by this process to synthesize the protein in its entirety outside the parasite. As the analysis with conformational epitope-recognizing monoclonal antibodies shows, the protein thus synthesized is at least reproducibly synthesizable over wide areas in naturally folded form. By the recombinant manufacturing process many milligrams of intact gp190/MSP1 could in every case be recovered from the host organism, a quantity which for

technical and economic reasons can never be recovered from parasites. Production of the protein in any desired quantity is now possible and opens new perspectives for its use as an experimental vaccine against malaria. Furthermore, the way is now open for the development of living vaccines as well as for vaccines based on nucleic acids.

Synthesis of the gene sequence coding for the protein gp190/MSP1 is preferentially based on the sequence of the FCB-1 strain of *P. falciparum*. *P. falciparum* is the agent of tropical malaria and hence of the most dangerous among the types of malaria. The basic gene is a representative of the "K1 allele", where K1 stands for a particular *P. falciparum* strain. Its coding sequence extends over 4917 base pairs and includes a signal sequence at the N-terminal end as well as an attachment sequence at the C-terminal end.

Furthermore, according to the invention the recombinant manufacturing process is preferentially characterized in having the AT content of the DNA sequence on which the protein is based reduced relative to the wild type, from 74% in the original gene preferably to about 55%, for example while the amino-acid sequence of the FCB-1 protein is maintained a DNA sequence with the codon frequencies usual in the human genome is produced. Other codon frequencies which reduce the AT content are also conceivable.

Preferentially the gene underlying the protein produced by the recombinant manufacturing process codes for the full amino-acid sequence including signal peptide and GPI attachment signal peptide, further described as gp190<sup>S</sup>.

In another preferred embodiment, the gene on which the protein produced by the recombinant manufacturing process is based codes for the complete amino-acid sequence except for the GPI attachment signal. This embodiment is then described as gp190<sup>S1</sup>.

In yet another preferred embodiment, the gene on which the protein produced by the recombinant manufacturing process is based codes for the complete amino-acid sequence except for the GPI attachment signal and the signal peptide. This embodiment is then described as gp190<sup>S2</sup>.

In a further preferred embodiment type, the gene on which the protein produced by the recombinant manufacturing process is based codes for the complete amino-acid sequence and a trans-membrane attachment sequence.

In a particularly preferred embodiment the recombinant manufacturing process includes the following steps:

In the first place the design of the DNA sequence to be synthesized on the basis of the gene from *P. falciparum* FCB-1, in which a DNA sequence with for example the codon frequencies common in the human genome is manufactured with retention of the amino-acid sequence of the FCB-1 protein.

The AT content of the gene should be reduced by this, preferably to 55%. Further on in the process the planned sequence is divided for example into 5 overlapping regions, which at the same time correspond to domains of the natural processing products of gp190/MSP1 from FCB-1: p83, p31, p36, p30 and p19.

Desoxyoligonucleotides are synthesized, which in each case extend the entire length of a region.

The desoxyoligonucleotides so synthesized are particularly preferred where their sequence corresponds in an alternating manner to the "upper" (5' - 3') or the "lower" (3' - 5') DNA strand. The length of these oligonucleotides is preferably on average 120 nucleotides and they overlap the neighboring sequences in each case by about 20 bases.

In one possible embodiment DNA sequences of about double the length of the existing end-products are manufactured by asymmetrical PCR, in effect so that the superfluous DNA sequences nearby in each case represent the opposite strand. This leads in a second

PCR amplification cycle to a second product corresponding to the length of four originally inserted oligonucleotides excluding the overlapping region. Transfer of these products to a preparation consisting predominantly of single-stranded DNA by asymmetrical PCR with the

terminal oligonucleotides permits the manufacture in a further amplification step of an 800-bp long double-stranded DNA fragment in only 25 PCR cycles.

In this manner the regions coding for p19, p30, p36 and p31 are directly synthesized and molecularly cloned in *E. coli*. Clones with fault-free sequences are conserved either directly or by the joining up of fault-free sequence fragments. The region which codes for p83 is constructed by fusion from two sequences comprising about 1200 bp.

In the further course of production single sequences are cloned. As expression vectors candidates preferred are the plasmids pDS56, RBS11 ("Hochuli, E., Bannwarth, W., Doebeli, H., Gentz, R. and Stueber, D. (1988) Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Biotechn.* 6, 1321-1325"), pBi-5 ("Baron, U., Freundlib, S., Gossen, M. and Bujard, H. (1995) Corregulation of two gene activities by tetracycline via a bidirectional promoter. *Nucl. Acids Res.* 23, 3605-3606") and ppTMCS. It is possible nonetheless also to conceive of other expression vectors.

Host organisms preferred for expression are *E. coli*, with the strain DH5alphaZ1 especially preferred (R. Rutz, Dissertation 1996, Heidelberg University), HeLa cells, CHO cells, *Toxoplasma gondii* (Pfefferkorn, E.R. and Pfefferkorn, C.C. 1976, *Toxoplasma gondii*: Isolation and preliminary characterization of temperature-sensitive mutants. *Exp. Parasitol.* 39, 365-376) or *Leishmania*. Additional host systems might be e.g. yeasts, baculoviruses or adenoviruses, so that the subject matter of the invention should not be limited to the host systems mentioned.

A further object of the present invention has been to provide a complete DNA sequence, suitable for expression, of the gp190/MSP1 surface protein of *P. falciparum*.

This object is solved by the invention named in Claim 17, by which the sequence can be obtained by the recombinant manufacturing process described above.

In a preferred embodiment of the present invention the sequence suitable for expression codes for the complete amino-acid sequence.

In another preferred embodiment of the present invention the sequence suitable for expression codes for the complete amino-acid sequence except for the attachment signal.

In a further preferred embodiment according to the present invention the DNA sequence suitable for expression codes for the complete amino-acid sequence except the attachment signal and the peptide signal. This embodiment of gp190/MSP1 can hence be characterized in including at the N-terminus 11 additional amino-acids, of which 6 are histidines.

Particularly preferred the DNA sequence suitable for expression contains no recognizable "splice-donor" and "splice-acceptor" sites, and is preferably characterized in not containing any larger GC-rich sequences which might result in stable hairpin structures at the RNA level.

Recognition signals for restriction enzymes which recognize sequences of six or more base pairs should preferably be avoided.

In a preferred embodiment specific cleavage sites for restriction endonucleases, occurring only once in the gene, are introduced into regions to separate the existing domains following processing of the protein.

Particularly preferred would be the presence at both ends of the gene of sequences for restriction endonucleases which do not occur in the gene.

Furthermore host organisms containing the complete sequence of gp190/MSP1 surface protein are provided by the invention.

Such host organisms are preferably *E. coli*, particularly preferred being the strain DH5alphaZ1, HeLa cells, CHO cells, *Toxoplasma gondii* or *Leishmania*. The HeLa and CHO cells ought preferably to synthesize constitutively tTA.

Finally the present invention provides a possibility of using a gp190/MSP1 surface protein created produced according to the recombinant manufacturing process, or parts thereof, for active immunization against malaria.

The scheme for synthesis presented here also permits manufacture of the second allele of the gp190/MSP1 gene, whereby the dimorphism of the protein is also taken into account. The main variability of the protein depends however on the sequences of two relatively short blocs, blocks II and IV (ref. 1), which are oligomorphic. The present sequence data make it possible to disclose over 95% of all known gp190/MSP1 sequences with 6-8 sequence combinations of these blocs. The synthesis of these sequence variants can be brought about problem-free by means of the strategies proposed here, so that variants can be built up both in the K1 and in the MAD20 allele. Vaccines from the families of sequences thus created can confer protection where required against a wide spectrum of parasites with gp190/MSP1 variants.

The manufacture of different types of vaccine is possible:

- At the level of protein preparations, where in each instance mixtures of the two families (K1 type, MAD20 type with different variants of Blocs II and IV) can come into use. Various carrier or adjuvant materials could be added: aluminum oxide, liposomes, IscomsQSz1, etc.
- At the level of live vaccines: (a) viral carriers, especially vaccinia and adenoviruses; (b) parasites as carriers, particularly avirulent forms of Leishmania and Toxoplasma; (c) bacterial carriers, e.g. Salmonella.
- At the level of nucleic acids, whereby for example vectors suitable for gene therapy would be used to introduce the gene into the host; beyond that the introduction of nucleic acids coding for the desired protein can be envisaged.

A further possibility for vaccination lies in the use of a gp190/MSP1 protein produced according to the recombinant manufacturing process set out by the invention, for the production of monoclonal antibodies which can then be used in their turn for passive immunization against malaria.



Similarly it becomes possible to use the DNA sequence on which the protein is based at an intermediate stage arising in the course of the recombinant manufacturing process for the construction of a vaccine based on nucleic acids.

Finally the invention also concerns a process for the stabilization of gene sequences, especially for sequences which do not show adequate stability in expression systems.

According to the invention this stabilization is attained because the AT content of the sequence is reduced.

Moreover a stabilized gene characterized by having a reduced AT content is provided by the invention. An example of such a stabilized gene is the gene for gp190/MSP1 surface protein according to the present invention.

In the following the invention will be described with the help of figures and tables as well as some examples in individual embodiments.

They show:

Fig. 1: Schematic representation of the gp190/MSP1 precursor protein from *P. falciparum* (FCB-1).

Fig. 2: Two vaccine trials carried out on Aotus monkeys with native gp190/MSP1 from *P. falciparum* (FCB-1).

Fig. 2A: With 3 x 60 micrograms gp190/MSP1

Fig. 2B: With 3 x 40 micrograms gp190/MSP1

Fig. 3A: Strategy of synthesis of the gp190/MSP1 gene

Fig. 3B: Principle of PCR-based total synthesis

Fig. 3C: Total sequencing of gp190<sup>S</sup>

Fig. 3D: N- and C-termini of gp190<sup>S1</sup> variant

Fig. 4A: Expression vector pDS56 with gp190<sup>S2</sup> sequence

Fig. 4B: Gel electrophoresis of gp190<sup>S2</sup>

Fig. 5A: Expression vector pBi-5 with gp190<sup>S1</sup> sequence

Fig. 5B: Immunofluorescence of HeLa cells

Fig. 5C: Electrophoretic characterization of gp190<sup>S1</sup>  
purified from HeLa cells

Fig. 6A: Expression vector ppT 190 with gp190 sequence

Fig. 6B: Immunofluorescence of the expression of gp190<sup>S</sup>  
in *T. gondii*

Fig. 6C: Polyacrylamide gel electrophoresis of gp190 from  
*T. gondii*

In the gp190/MSP1 precursor protein from *P. falciparum* schematically represented in Fig. 1 the dark blocs stand for regions which are strongly conserved in all strains. The cross-hatched blocs indicate the dimorphic areas, which in the case of the FCB-1 isolate derive from the K1 allele. O1 and O2 indicate the oligomorphic areas. S denotes the peptide signal sequence containing 19 amino-acids, GA the C-terminal region, which includes the signal for the GPI attachment of the protein to the membrane. The arrows indicate the sites of the processing by

which the proteins p53, p31, p36, p30 and p19 arise. The gp190 gene codes for altogether 1639 amino-acids.

The other figures are more conveniently explained in the context of the following Examples.

## EXAMPLES

**Example 1:** total synthesis of one of the DNA sequences coding for gp190/MSP1 (see Fig. 3)

### A. Strategy of synthesis of the gp190/MSP1 gene (gp190<sup>S</sup>) (see Fig. 3A).

The sequence was divided into fragments corresponding to the main processing products: p83, p31, p36, p30 and p19. In the transition regions cleavage sites for restriction endonucleases (arrows in fig.3) were inserted in such a way that the amino-acid sequence was not altered. All the particular cleavage sites are found only once in the sequence.

The fragments were synthesized to overlap, so that the cleavage sites at the respective ends made attachment by ligation to the neighboring fragment possible. All individual fragments contain in addition at their 5' ends a BamHI division site for insertion into expression vectors. The entire sequence could be cloned via MluI and ClaI. The scheme indicated here leads in addition to a sequence which cannot produce the GPI attachment since the C-terminal lacks 18 amino-acids. Synthesis of a corresponding oligonucleotide as well as of a "primer" extending over the SphI cleavage site, leads after PCR to the GA fragment which could be used by SphI and ClaI, the resulting total sequence being gp190<sup>S</sup>. On removing the sequence coding for the peptide signal, "PCR Primer" is produced, over which the fragment  $\Delta S$  has been synthesized. It is permissible to alter the N-terminal via a BamHI and a HindIII cleavage site in such a way that the protein begins with amino-acid no. 20. The nuclear sequence which encodes gp190/MSP1 without signal sequence and without GPI attachment signal was designated gp190S2. Deletion of the GPI attachment signal alone leads to gp190<sup>S1</sup>.

## B. Principle of the PCR-supported total synthesis (see Fig. 3B)

Oligodesoxynucleotides of about 120 nucleotides have been synthesized in an alternating manner from the coding or the non-coding strand in such a way that in each case about 20 bases overlapped with the neighboring fragment. The scheme illustrates for example the synthesis of a fragment about 800bp long from oligonucleotides. At the first stage 2 oligonucleotides were amplified "asymmetrically" in each of 4 reaction vessels. This resulted in 4 populations of DNA about 220bp in length, consisting predominantly of single strands (A, B, C, D). Uniting A to B and C to D with amplification over 5 cycles led to 2 approximately 400bp long double-stranded products. Asymmetrical amplification of these DNA fragments (Stage III) resulted in single-stranded populations which following uniting and amplification (Stage IV) resulted after 10 cycles in the end-product G of about 800bp in length. This synthesis could be carried out without isolation of intermediate products and without renewing buffer or enzyme, and was completed in 3 hours. The end-product was purified electrophoretically, divided up with the appropriate restriction endonucleases, and cloned in *E. coli* in pBluescript (Stratagene), to which polylinker a MluI and a ClaI cleavage site had been added.

## C. Total sequence of gp190<sup>S</sup> (see Fig. 3C)

Following fusion of all part sequences (Fig. 3A) in pBluescript, the sequence of the gene was checked by the di-deoxy method. The reading frame of gp190<sup>S</sup> had a length of 4917bp (+ 2 stop-codons) and encoded an amino-acid sequence corresponding to that of the gp190/MSP1 from FCB-1 (1639 amino-acids).

## D. N- and C-termini of the gp190<sup>S1</sup> variant (see Fig. 3D)

The N-terminal sequence, beginning with the BamHI cleavage site, indicates the transition at amino-acid 20, from which it can be assumed that after splitting of the signal peptide it defines the N-terminus. At the C-terminus the sequence encoded ended at amino-acid 1621. The stop-codon followed the ClaI cleavage site.

## Example 2: Expression of gp190<sup>S2</sup> in E. coli

### A. Expression vector (see Fig. 4A)

The gp190<sup>S2</sup> sequence was inserted via the BamHI and Cial cleavage sites into pDS56RBSII, by means of which 6 histidines as well as some amino-acids originating in the vector were fused to the N-terminus. This produces the following N-terminal sequence on the reading-frame: Met Arg Gly Ser (His)<sub>6</sub> Gly Ser. Through the promoter P<sub>N25lac0-1</sub> the transcription comes under lacR/O/IPTG control.

### B. Expression and purification of gp190<sup>S2</sup> (see Fig. 4D)

Carrying over the vector pDS56RBSIIgp190<sup>S2</sup> into E. coli DH5alphaZ1 and induction of synthesis through IPTG resulted after electrophoretic separation of the total protein extract from the culture in a clearly visible band of the anticipated size (arrow). Purification of the material through IMAC and affinity chromatography (antibody column with mAK5.2) led to a homogeneous product of about 190 kD. In the Figure M stands for molecular weight standards; 1 = E. coli before; 2 = after induction with IPTG for 2 hours; 3, 4, 5 = fractions from elution of the mAK column.

## Example. 3: Tetracycline-controlled expression of gp190<sup>S1</sup> in HeLa and CHO cells and isolation of the product (see also Fig. 5 and 6c)

A. The gp190 sequence was inserted via the BamHI/ClaI cleavage sites into the expression vector pBi-5. In this way transcription of the gene came under the control of a bidirectional "tTA-reponsive" promoter and could be regulated through Tc. The bidirectional promoter simultaneously initiated transcription of the indicator gene luciferase. In consequence the regulation of the expression could easily be followed (see also Fig. 5A).

### B. Immunofluorescence of HeLa cells, which express luciferase and gp190<sup>S1</sup> under Tc control

The production of luciferase (left), gp190<sup>S1</sup> (middle) in the absence of Tc was demonstrated in HtTA93-9 cells, which contain the bidirectional transcription unit of (A). Following addition of Tc no noteworthy synthesis of gp190<sup>S1</sup> was shown (as represented in Fig. 5B,right).

### C. Electrophoretic characterization of gp190<sup>S1</sup> purified from HeLa cells

The HeLa cell clone HtTA93-9 as well as the CHO cell clone CHO27-29 have been cultivated with or without Tc. Cell extracts separated by electrophoresis have been analyzed with mAK5.2 by means of "Western blotting" (Fig. 5C); analysis of the CHO cell line is shown on the left, of HeLa on the right. (1) = culture without, (2) = culture with Tc, (3) = non-transfected HtTA-1 cell line. Molecular weight standards are in each case indicated on the left.

### D. Purification of gp190<sup>S1</sup> synthesised by HeLa cell clone HtTA93-9

Preparative cultivation of the HtTA line and induction of expression of gp190<sup>S1</sup> by withholding Tc permitted isolation of the gene product by affinity chromatography (mAK5.2 column).

The polyacrylamide gel stained with Coomassie (Fig. 6C) following electrophoresis displayed a product consisting of gp190<sup>S1</sup> as well as another protein of about 50 kD. The latter was not derived from gp190<sup>S1</sup> and thus originated from the HeLa cells. Its projected removal should nevertheless present no difficulty in principle.

### Example 4: Expression of gp190<sup>S1</sup> in Toxoplasma gondii and purification of the product (see also Fig. 6).

A. The gp190<sup>S</sup> sequence was inserted into the vector ppT via MluI/PstI. This brought the gene under the control of the tubulin promoter ( $P_{\text{tub-1}}$ ) of *T. gondii*. The 3' untranslated region (VTR) originated from the main surface protein of *T. gondii* (SAG-1).

### B. Expression of gp190<sup>S</sup> in *T. gondii*

Transfection of *T. gondii* with pTT190 led to the isolation of parasite lines which expressed constitutively gp190<sup>S</sup>. Immunofluorescence with mAK5.2 (middle picture) showed not only expression of the gene but also situated the binding of the expression product close to the surface of the parasite, since it, like SAG-1, provokes the same pattern of immunofluorescence (right section of fig. 6B). On the left in Fig. 6B a phase contrast photograph of the middle picture is shown.

### C. Isolation of gp190<sup>S</sup> from *T. gondii*.

By means of affinity chromatography (mAK5.2 column) gp190<sup>S</sup> was purified from a prepared quantity of *T. gondii* ( $5 \times 10^9$  parasites). The extremely pure protein possessed the anticipated molecular weight, as the Coomassie-stained polyacrylamide gel indicated following electrophoresis (2-3 on Figure 6C). At no. (1) on Fig. 6C purified gp190<sup>S1</sup> from CHO cells is represented with molecular weight marked on the left side.

### Example 5: Characterization of gp190<sup>S</sup> with monoclonal antibodies.

The interaction of 16 monoclonal antibodies with gp190<sup>S</sup> from the various heterologous expression systems was reviewed by immunofluorescence on *P. falciparum* and *T. gondii* or by "Western blot" on the purified proteins. Complete agreement was found when the two parasites were compared (number of +s indicates the relative intensity of the fluorescence). On Western blotting 12mAK's reacted with gp190<sup>S</sup> from *E. coli* and *T. gondii*. On the other hand 3 antibodies did not bind to material isolated from CHO cells. Antibodies 15 and 16, which recognize epitopes from the oligomorphs or the alternative allele (MAD20), did not react. The results are summarized in Table 1, in which ND means "not carried out".

- Unsecreted foreign proteins at high concentration can interfere negatively with cell metabolism. Synthesis of the desired product is consequently begun only after maturation of the culture.



In the construct pBi5-gp190<sup>S1</sup> a bidirectional promoter was activated by the Tc-controlled transcription activator and initiated transcription of both gp190<sup>S1</sup> and the luciferase indicator gene. In the presence of Tc the promoter is inactive. The transcription unit was transferred into both HeLa and CHO cells, which both synthesize constitutively tTA (HtTA line: Gossen, M. and Bujard, H. (1992), Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA 89, 5547-5551; CHO-tTA line, unpublished). Through cotransfection (Ca<sup>2+</sup>-phosphate method) with a hygromycin-resistance-inducing marker gene was selected for successful chromosomal integration. Hygromycin-resistant clones were then investigated for regulability of the expression >Tc, in which luciferase activity was used as indicator. The gp190 synthesis was tested in well regulable clones (regulation factor •Tc 1000). Immunofluorescence analysis (Fig. 5B) as well as investigation by "Western blot" (Fig. 5C) allowed the identification in both cell types of clones which synthesized gp190 under strictly regulable conditions. The best regulable of 20 clones were in each case subcloned. The subclones HtTA93-9 and CHO27-29 were used for cultures on a scale of 10:1. From cell extracts of these cultures intact gp190<sup>S1</sup> could be isolated by means of affinity chromatography (mAK5.2). The material was homogeneous except for a single cellular component which did not derive from gp190<sup>S1</sup> and made up 25% of the preparation (Fig. 6C). It had to be removed in a further purification step.

### 3. Expression of gp190S in Toxoplasma gondii.

Like *P. falciparum*, *Toxoplasma gondii* belongs to the Apicomplexa and consequently has a protein modification system apparently similar to that of *P. falciparum*. *T. gondii* can be transfected with foreign DNA which is efficiently integrated into the genome and furthermore allows problem-free multiplication of *T. gondii* in cell culture. To obtain a product most like native gp190, gp190S2 is expressed in such a way that the protein is secreted on the surface of the parasite and, as in *P. falciparum*, bound to the membrane via a GPI analogue. In that way the gp190S2 (Fig. 3A) has been inserted (Fig. 6A) into the plasmid ppTMCS (D. Soldati, unpublished) and thereby placed under the control of the *T. gondii* tubulin promoter,

This expression construct was transfected into *T. gondii*. Selection with chloramphenicol led to resistant clones synthesizing gp190 which were detected by immunofluorescence (Fig. 6B).

The immunofluorescence with anti-gp190 antibodies was indistinguishable from a corresponding pigmentation of the parasites by means of antibodies against SAG1, the main surface protein of *T. gondii*. It may be deduced from this that gp190 is bound to the surface of *T. gondii*. Several *T. gondii* clones (Nos. 3.1 to 3.4) were characterized and saved for the production of gp190. Using affinity chromatography (mAK5.2) gp190 was isolated from *T. gondii* cultures (clone 3.4) cultivated on a preparative scale. Electrophoretic analysis revealed a homogeneous product with a migration rate similar to that of the intact protein (Fig. 6C).

Example 7: Characterization of gp190 protein from various expression systems by means of monoclonal antibodies.

A set of gp190-specific monoclonal antibodies, of which a number recognize conformational epitopes, were used to compare the reactivity of the antibodies with *P. falciparum* and *T. gondii* parasites via immunofluorescence. Table 1 shows that the reactivity of the 16 antibodies with either parasite is the same. This is a strong indication that in *T. gondii* "native" gp190 is being mostly produced. Comparison of the reactivity of the antibodies with protein from *E. coli*, HeLa or CHO cells as well as *T. gondii* shows also that most of the antibodies react with the 4 preparations. In particular the protein derived from *E. coli* recognizes more of the antibodies than that produced in mammalian cells. This is apparently a consequence of glycosylation in mammalian cells.

Example 8: Immunization of *Aotus lemurinus griseimembra* monkeys with gp190/MSP from *P. falciparum* (FCB-1).

Two independent immunization experiments (A, B) were carried out. In them in one instance (A) 1.0 mg and in the other (B) 0.6 mg of very pure gp190/MSP1 was extracted from about  $2 \times 10^{11}$  parasites respectively.

The protein was administered together with Freund's Adjuvant (FCA). The control group received only FCA. Immunization equally with the protein mixture or the adjuvant was done three times at intervals of 4 weeks. Two weeks after the last immunization each of the animals

was infected with  $10^5$  parasites (FVO strain) from a donor animal. Parasitaemia was measured daily. The results are summarized in Fig. 2. The symbols mean:

T: that the animals were treated with resochin

D: a dead animal

Fig. 2A: individuals in the vaccinated group each received  
3 x 60 micrograms gp190/MSP1

Fig. 2B: individuals in the vaccinated group each received  
3 x 40 micrograms gp190/MSP1

While in the control group only 1/11 animals did not develop parasitaemia, this was 6/10 in the vaccinated group. The four animals in the vaccinated group who did develop a pronounced parasitaemia did so - in comparison to the control group - with an average delay of four days (exceeding the 2% limit of parasitaemia).

These experiments indicate for the first time a highly significant protection by gp190/MSP1 against infection with *P. falciparum* in a monkey model. The process according to the invention consequently permits a practical vaccine against malaria to be presented for the first time.

Table 1: Interaction of gp 190<sup>s</sup> with monoclonal antibodies

Code	mAb	Type of epitope	Variability	P.f. FCB	IFA		Western blot	
					Toxoplasma	E. coli	Toxoplasma	CHO
1	5.2	conformational	conserved	++++	++++	+	+	+
2	12.10	conformational	conserved	++++	++++	+	+	+
3	7.5	conformational	conserved	++++	++++	+	+	+
4	12.8	conformational	conserved	++	++	+	+	+
5	7.3	conformational	dimorph (K1)	++++	+++	+	+	+
6	2.2	conformational	conserved	++++	++++	+	+	+
7	7.6	conformational	dimorph (K1)	++++	++++	+	+	+
8	9.8	conformational	conserved	++++	++	+	-	-
9	13.2	sequential	conserved	++++	++++	+	+	+
10	13.1	sequential	dimorph (K1)	++++	+++	+	-	-
11	6.1	sequential	dimorph (K1)	++++	++++	+	+	ND
12	A5Z	unknown	unknown	+++	+++	+	+	+
13	17.2	unknown	unknown	++++	+++	ND	ND	ND
14	15.2	unknown	unknown	++++	+++	ND	ND	ND
15	9.7	conformational	dimorph (MAD20)	-	-	-	-	-
16	12.1	sequential	oligomorph	-	-	-	-	-

## Patent Claims

1. Process for preparing of the complete gp190/MSP1 protein of Plasmodium, in particular Plasmodium falciparum characterized in that the complete gene for gp190/MSP1 is expressed in a suitable system, preferably a host organism.
2. Recombinant manufacturing process according to Claim 1, characterized in that the synthesis of the DNA sequence on which the protein is based derives from the DNA sequence of the P. falciparum strain FCB-1.
3. Recombinant process according to Claim 2, characterized in that the AT content of the DNA sequence expressed is reduced compared to that of the naturally occurring sequence, preferably from 74% to 55%.
4. Recombinant process according to one or more of claims 1 to 3, characterized in that the gene on which the protein produced is based codes for the complete amino-acid sequence including signal peptide and attachment signal.
5. Recombinant process according to one or more of claims 1 to 3, characterized in that the gene on which the protein produced is based codes for the complete amino-acid sequence except for the attachment signal.
6. Recombinant process according to one or more of claims 1 to 3, characterized in that the gene on which the protein produced is based codes for the complete amino-acid sequence except for the attachment signal and the signal peptide.
7. Recombinant process according to one or more of claims 1 to 6, characterized in that it includes the following steps:
  - (a) Design of the DNA sequence from P. falciparum FCB-1 to be synthesized, by which a DNA sequence with the codon frequencies common in the human

genome and maintaining the amino-acid sequence of the FCB-1 protein would be produced,

- (b) division of the planned sequence into overlapping regions, preferably regions p83, p31, p36, gp30 and gp19,
  - (c) synthesis of desoxyoligonucleotides, each of them extending the whole length of a region,
  - (d) synthesis of the regions coding for gp19, gp30, p36 and p31 by PCR and synthesis of the region coding for p83 by fusion of two sequences comprising approximately 1200 bp,
  - (e) individual cloning of coding sequences,
  - (f) fusion of the complete gene and
  - (g) expression in a suitable expression system.
8. Recombinant process according to Claim 7, characterized in that the desoxyoligonucleotides synthesized in step (c) should be on average 120 nucleotides long and in each instance overlap the neighbouring sequences by about 20 bases.
  9. Recombinant process according to one or more of Claims 1 to 8, characterized in that dPS56, RBSII is used as expression vector.
  10. Recombinant process according to one or more of Claims 1 to 8, characterized in that pBi-5 is used as expression vector.
  11. Recombinant process according to one or more of Claims 1 to 8, characterized in that ppTMCS is used as expression vector.

12. Recombinant process according to one or more of Claims 1 to 11, characterized in that the DNA sequence on which the protein is based is expressed in *E. coli*.
13. Recombinant process following Claim 12, characterized in that the *E. coli* strain used is the repressor-producing strain *E. coli* DH5alphaZ1.
14. Recombinant process according to one or more of Claims 1 to 11, characterized in that the DNA sequence on which the protein is based is expressed in HeLa cells.
15. Recombinant process according to one or more of Claims 1 to 11, characterized in that the DNA sequence on which the protein is based is expressed in CHO cells.
16. Recombinant process according to one or more of Claims 1 to 11, characterized in that the DNA sequence on which the protein is based is expressed in *Toxoplasma gondii* or *Leishmania*.
17. Complete DNA sequence, suitable for expression, of the gp190/MSP1 surface protein of *Plasmodium*, in particular *P. falciparum*, preferably obtainable through the recombinant process according to one or more of Claims 1 to 16.
18. DNA sequence, suitable for expression, according to Claim 17, characterized in that it does not code for the attachment signal.
19. DNA sequence, suitable for expression, according to Claim 18, characterized in that it codes neither for the attachment signal nor the signal peptide.
20. DNA sequence, suitable for expression, according to Claim 19, characterized in that it includes a sequence for 11 additional amino-acids, of which 6 are histidines, present at the N-terminus.

21. DNA sequence, suitable for expression, according to one or more of Claims 17 to 20, characterized in that the sequence includes no recognizable "splice donor" and "splice acceptor" signals.
22. DNA sequence, suitable for expression, according to one or more of Claims 17 to 21, characterized in that the sequence includes no large GC-rich sequences.
23. DNA sequence, suitable for expression, according to one or more of Claims 17 to 22, characterized in that the sequence includes no recognition signals for restriction enzymes recognizing sequences of six or more base pairs.
24. DNA sequence, suitable for expression, according to one or more of Claims 17 to 23, characterized in that the sequence for recognition signals of particular restriction nucleases in regions which, after processing of the protein, separate existing domains, contains uniquely occurring cleavage sites for restriction endonucleases.
25. DNA sequence, suitable for expression, according to one or more of Claims 17 to 24, characterized in that the sequence has at both its ends cleavage sites for endonucleases which do not appear in the remaining sequence and in a vector for use.
26. Host organism which contains the complete nucleic acid sequence for the gp190/MSP1 surface protein and/or the complete protein.
27. Host organism according to Claim 26, characterized in that the organism is *E. coli*.
28. Host organism according to Claim 27, characterized in that the *E. coli* strain is the repressor-producing *E. coli* strain DH5alphaZ1.
29. Host organism according to Claim 26, characterized in that the host organism is HeLa cells.



30. Host organism according to Claim 26, characterized in that the host organism is CHO cells.
31. Host organism according to Claim 29 or 30, characterized in that the host cells synthesize constitutively tTA.
32. Host organism according to Claim 26, characterized in that the hosts provided are Toxoplasma gondii, Leishmania, baculoviruses, adenoviruses or yeasts.
33. Use of a gp190/MSP1 protein manufactured according to Claims 1 to 16 for active immunization against malaria.
34. Use of a gp190/MSP1 protein manufactured according to Claims 1 to 16 for the manufacture of monoclonal antibodies suitable for passive immunization.
35. Use of a DNA sequence manufactured according to Claims 1 to 16 for the manufacture of a vaccine based on nucleic acids.
36. Process for stabilization of gene sequences, characterized in that the AT content of the sequence is reduced.
- ~~37.~~  
38. Stabilized gene, characterized in that it shows a reduced AT content than the unstabilized gene.
- ~~38.~~  
39. Vector containing a DNA sequence following one of the Claims 17 to 25 and/or ~~36.~~<sup>37.</sup>
- ~~39.~~  
40. Host cells containing a vector according to Claim ~~37.~~<sup>38.</sup>
- ~~40.~~  
41. Vaccine containing a protein manufactured according to a process according to any of the Claims 1 to 16 and/or a DNA sequence according to one of the Claims 17 to 25 and/or a host according to one of the Claims 26 to 32 and/or a vector according to Claim ~~37.~~<sup>39.</sup>

653030 42363660

41.  
~~40.~~

40

Vaccine according to Claim ~~39~~, characterized in that it contains further immunity-promoting products from Plasmodium, especially *P. falciparum*.

66000 42369669

## Summary

Recombinant manufacturing process for a complete malaria antigen gp190/MSP1

The invention concerns a recombinant manufacturing process for the complete gp190/MSP1 surface protein of Plasmodium, in particular *P. falciparum*, as well as the complete DNA sequence of this protein and suitable host organisms for expression of the sequence, by means of which the protein in its entirety can for the first time be synthesized outside the parasite.

The invention opens up for the first time the possibility of manufacturing the gp190/MSP1 surface protein in sufficient quantity; in addition there is the matter of making gp190/MSP1 available as a vaccine.

Finally the invention sets out a process for the stabilization of AT-rich genes (Fig. 1).

66000"42869260

Fig. 1

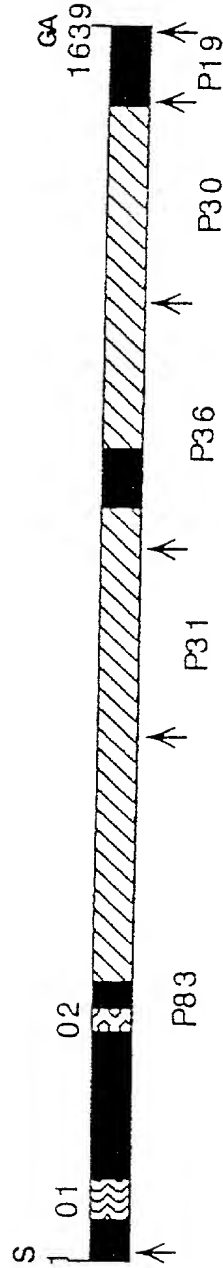
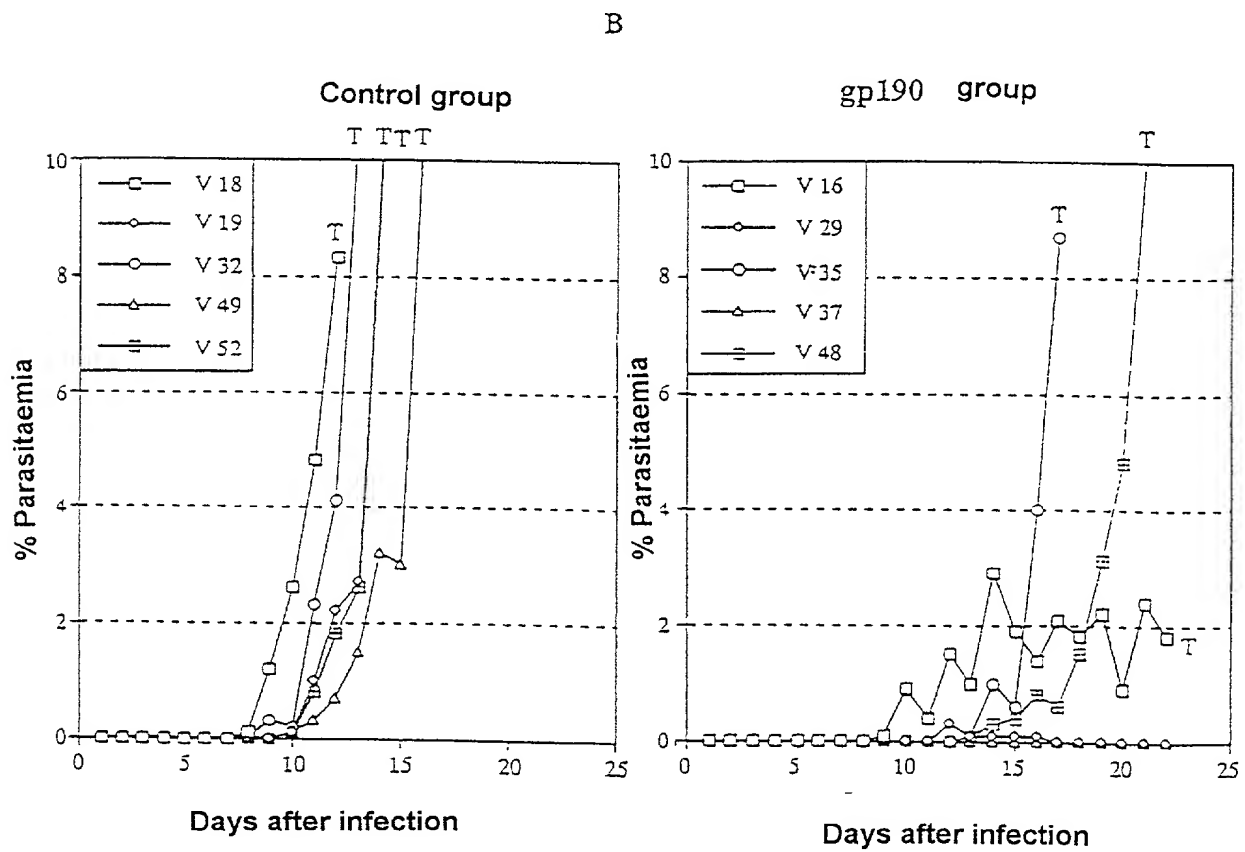
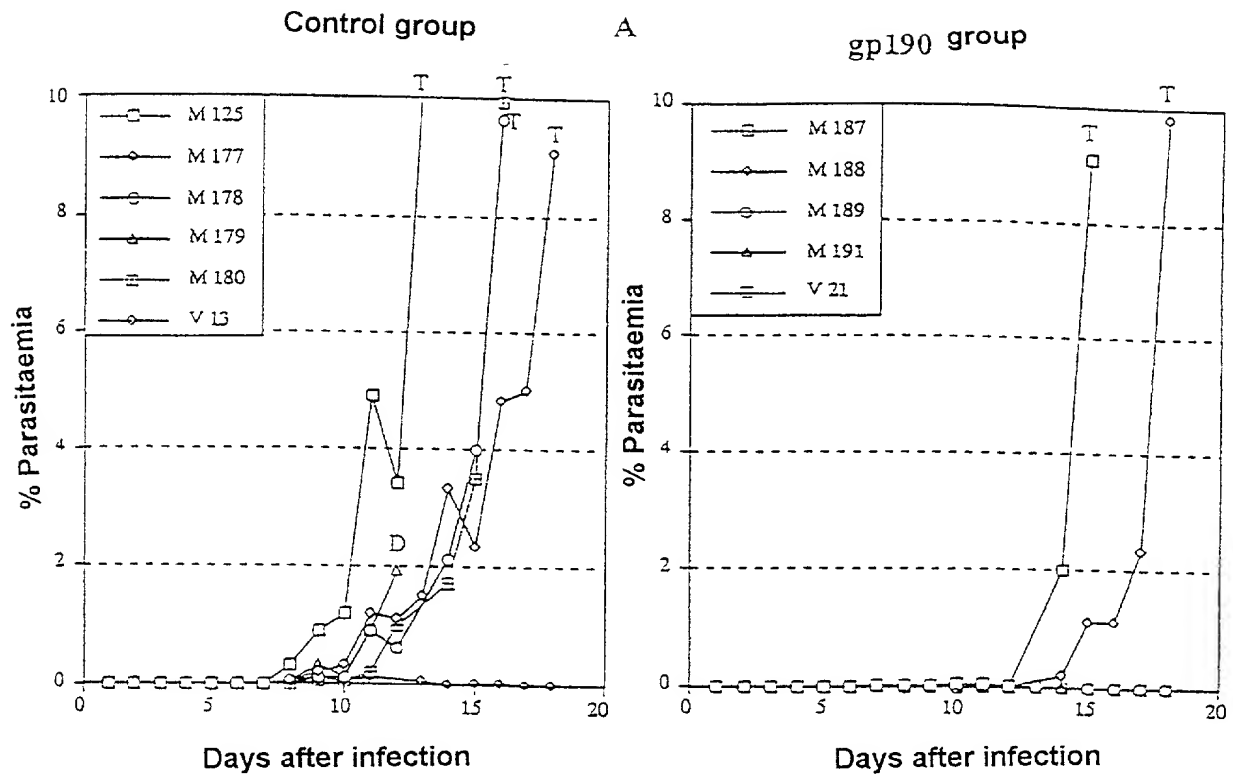
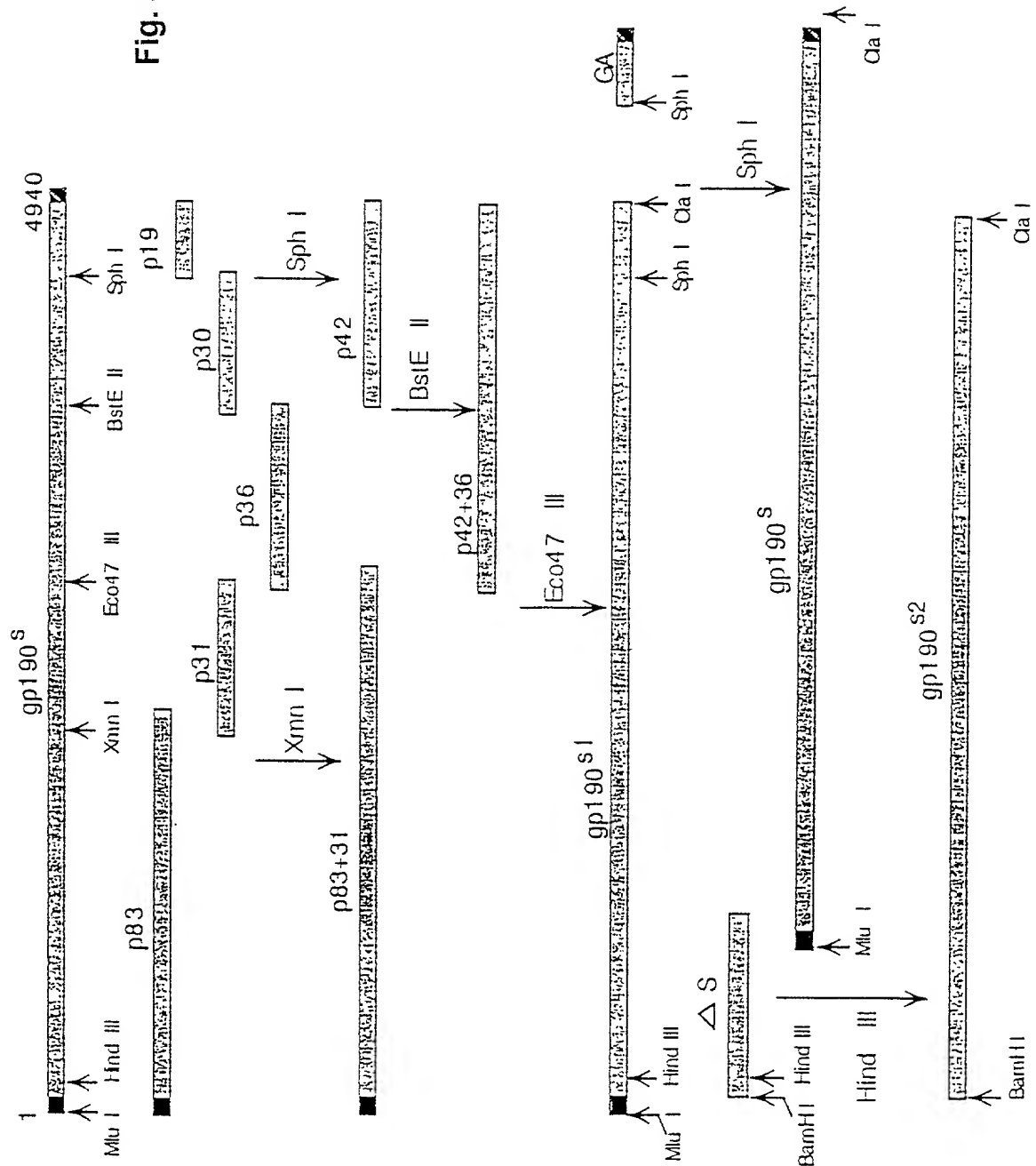


Fig. 2





**SECRET**

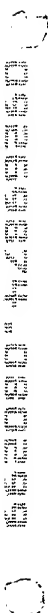


Fig. 3C

5/16

DNA sequence of the native (gp190n) and of the synthetic gene (gp190s) for gp190 from FCG-1

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AS      M K I I F F L C S F L F F I I N T Q C V T H E S Y Q E      27
gp190n  G A A T T A T T A A A T A A T A A T A A A A A A A A A A
gp190s  CGCAGCGGATGAAAATCATTTCTCTCTGTCATTCTGTTTTTATCATCAATACTCAGTCCGTGACCCAGAACTCTATCAGGAG
      90
      H I I
AS      L V K K L E A L E D A V L T G Y S L F Q K E K M V L N E G T      57
gp190n  T C A A A A A A A T G A T T T T A T A A A A A T A A A A
gp190s  CTGGTTAAGAAACTGGAAGCTTTGGAAGATGCCGCTCTTACCGGATACAGCCTGTTCCAGAAGGAGAAGATGGTCTGAATGAAGGGAGC
      180
AS      S G T A V T T S T P G S K G S V A S G G S G G S V A S G G S      87
gp190n  A A T T T T A G T A T T C A T A C A T T A T C A
gp190s  AATGGCAGCCCGCTTACACACGACACCCCGTTCTAAAGGGTCTGTGGCTAGCCGGTGGCTCCGGTCCGCTCTGTGGCTCTGGGGGTTCC
      270
AS      V A S G G S V A S G G S V A S G G S G N S R R T N P S D N S      117
gp190n  T T A T C A T T T T C A T T A T T C A A C T A T A T A T A
gp190s  GTCCCTCCGGCGGCGGCTGGCATCAGGTGGCTCAGTGGCAAGCGCGGTTCCGGGAACAGTCGAAGAACCATCCATCTGACAACCTCT
      360
AS      S D S D A K S Y A D L K H R V R N Y L L T I K E L K Y P Q L      147
gp190n  T A T T A T T T A A A A C T C T G T A A A C A T T A C C
gp190s  AGCGATTCCGAGCCCAAGTCTCTACGCGGACCTCAAGCAGCGAGTGAAGAACTATCTCTCCTACTATCAAGGAGCTGAAGTACCCACAGTTG
      450
AS      F D L T N H M L T L C D N I E G F K Y L I D G Y E E I N E L      177
gp190n  T T A T A T T T T T T T T T A A T A T A T A T A T A T A
gp190s  TTGACCTCACTAATCATATGCTGACACTGTGTGATAACATTGCTGGCTTCAATATCTGATTGACGGTTACGAAGAGATCAATGAATCTC
      540
AS      L Y K L N F Y F D L L R A K L N D V C A N D Y C Q I P F N L      207
gp190n  T A T A A C T T T T T A T A A T A T A T A T A T A T A T A
gp190s  CTGTACAAGTTGAATTTCTACTTCGACTTGCTAAGGGCCAAACTGAATGAGCTTTGGCCCAATGACTATTGTCAAATTCATTCATTTG
      630
AS      K I R A N E L D V L K K L V F G Y R X P L D N I K D N V G X      237
gp190n  A T C T A T A A C T A A C T G T A A A A A T A T A T A T A
gp190s  AAGATCAGAGCCCAAGGAGTGGAGCTAATGAAGAAGTGGTCTTCGGATATCGCAAGCCTCTCGACAACATCAAGGACAATGTGGGAAG
      720
AS      H E D Y I K K N K K T I E N I N E L I E E S K K T I D K N X      267
gp190n  C A A A A A A T A T A T A T A T A T A T A T A T A T A
gp190s  ATGGAAGATTATATTAAAAAATAAAGAACCATCGAGAACATTACGAGCTGATCGAAGAATCCAAAAAGACCATAGACAAAAATAAG
      810
AS      N A T K E E E K K K L Y Q A Q Y D L S I Y N K Q L E E A H N      297
gp190n  T A A A A A A A A T A T T T T T T C T A T A T A T A T
gp190s  AATGCAACCAAGGAGGAAGAAAAGAAAGTTGTACCAGGCCAGTACGACCTGTCCATCTATAACAAACAGCTTGAGAAGCCCATAC
      900
AS      L I S V L E K R I D T L K K N E N I K E L L D K I N E I K N      327
gp190n  T A A T T A A A T T T T A A A C T G T A T T A A A
gp190s  CTCATCAGCGTACTCGGAGAGCCCATGACACCCCTCAAGAAGAATGAAATATCAAGAAGTCTGCGACAAGATTAATGAATTAAGAAT
      990
AS      P P P A N S G N T P N T L L D K N K K I E E H E K E I K E I      357
gp190n  C A G T A T A A T T C T T A A C A A A A T A A T
gp190s  CCTCCGCCAGCCCACTCTCGGAACACCCCTAACACCGCTCTCGACAAAGAACAGATAGAGGAGCAGGAGAAAGAGATCAAGAGATC
      1080
AS      A K T I K F N I D S L F T D P L E L E Y Y L R E K N K N I D      387
gp190n  T A T T T A G T A A A T A A T A A A A A A A T T
gp190s  GCCAAAACCATTAAGTTCAACATAGATTCTCTCTTACTGATCCCTTGAGCTGGAGTACTACTTGAGAGAGAAGAATAAGAATATAGAC
      1170
AS      I S A K V E T K E S T E P N E Y P N G V T Y P L S Y N D I N      417
gp190n  A A G T A G T A T C A A A A A A A A T T T T A T
gp190s  ATCTCCGCCAAAGTCGAGACAAAGGAATCAACCGAACCCTAATGAATATCCCAATGGTGTGACGTACCCCTCTGTCTTATAACGATATCAAC
      1260
AS      N A L N E L N S F G D L I N P F D Y T K E P S K N I Y T D N      447
gp190n  T T A T A T T C T T T A A T A T A A A A A A A A A C A C A T T T
gp190s  AACGCTCTCAACGAGCTCAATAGCTTCGGTGACTTGATTAAACCCCTTCGATTATACGAAAGAACCCTCTAAGAATATCTACACAGACAAT
      1350
AS      E R K K F I N E I K E K I K I E K K K I E S D K K S Y E D R      477
gp190n  A A C A T T A A T A A A A A A A A T C T A T C
gp190s  GAGAGAAGAAGTTTATCAACGAAATCAAGGAGAAGATCAAAATTCAGAAGAAGAAAATTCAGAGCTGACAAGAAAAGTTACGAAGACCCG
      1440
AS      S K S L N D I T K E Y E K L L N E I Y D S K F N N N I D L T      507
gp190n  T C T G T C T T A A A A A T A T T A T A T A G T T A T T A T
gp190s  AGCAAAAGCTTAACGATATCACTAAAGAGTATGAAAAGCTGCTGAACGAGATCTATGATTCCAAATTCACAAATACATCGACCTGACC
      1530
AS      N F E K H M G K R Y S Y K V E K L T H H N T F A S Y E N S X      537
gp190n  T A T A T A T T T T T T T T T T T T A A A A
gp190s  AACTTCGAGAAAATGATGGGAAAACGGTACTCTTACAAAGTGGAGAAAATGACACACCATAATACCTTTGCATCTCTATGAGAATCTAAG
      1620

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AS H N L E K L T K A L K Y M E D Y S L R N I V V E K E L K Y Y 567  
gp190<sup>n</sup> A T A A A A T T A A T A A T A T A T 1710  
gp190<sup>s</sup> CATAATCTTGAGAAGCTCACCAGCTCTTAAGTATATGGAGGACTATTCTCTCGGAACATTGTTGTGGAGAAGAACTAAAGTATTAC

AS K N L I S K I E N E I E T L V E N I K K D E E Q L F E K K I 597  
gp190<sup>n</sup> A T A C A A T T A A T A A T A A C T A A A 1800  
gp190<sup>s</sup> AAGAATCTCATAAGTAAGATCGAAAACGAGATCGAGACGCTTGTGAGAACATTAAGAAGGATGAAGAACAGTTGTTTGAGAAGAAGATT

AS T K D E N K P D E K I L E V S D I V K V Q V Q K V L L M N K 627  
gp190<sup>n</sup> T A A T T A A A T C A A T A A T T A T A A 1890  
gp190<sup>s</sup> ACAAAACACGAAAATAAACAGATGAGAGATCCTGGAGGTCCTCCGATATTGTTAAAGTCCAAGTGCAGAAGGTGCTCCTCATGAACAAG

AS I D E L K K T Q L I L K N V E L K E N I H V P N S Y K Q E N 657  
gp190<sup>n</sup> C T A A T G T A A T A A T C T C C A A A 1980  
gp190<sup>s</sup> ATTGATGAACCTCAAGAAGACTCAACTCATCTGGAAGAACGTGGAGTTAAACATTAACATACATGTGCCGAATAGTTATAAGCAGGAGAAT

AS K Q E P Y Y L I V L K K E I D K L K V F M P K V E S L I N E 687  
gp190<sup>n</sup> A T T T T A T G T G A A T T T A T G A A T C A T A T 2070  
gp190<sup>s</sup> AAGCAGGAACCATACTACCTCATCTGACTCAAGAAAGATAGACAACTGAAAGTGTTCATGCCCAAGTGCAGAGGCTTATCAACGAA

AS E K K N I K T E G Q S D N S E P S T E G E I T G Q A T T K P 717  
gp190<sup>n</sup> A A A A A T A G T G A A A C A A A A A T A A T 2160  
gp190<sup>s</sup> GAGAAGAAGAACATTAAGCTGAAGGACAGTCAGATAACTCCGAGCTTCCACAGAGGAGAGATAACCGGACAGGCTTACACCAAGCCCC

AS G Q Q A G S A L E G D S V Q A Q A Q E Q K Q A Q P P V P V P 747  
gp190<sup>n</sup> A A A A T T A T C A A A A A A A A A A A A 2250  
gp190<sup>s</sup> GGACAAACAGGCGGTTCCAGCTCTCGAAGGCGATAGCGTGCAGCTCAAGCACAAGAGCAGAAGCAGGACAGGCTTCCAGTGCCTGCCCC

AS V P E A K A Q V P T P P A P V N N K T E N V S K L D Y L E K 777  
gp190<sup>n</sup> A A A A C A A A A A T A T A T T C T A T T A A 2340  
gp190<sup>s</sup> GTTCCAGAGGCTAAAGCTCAAGTCCCTACACCACCGCTCTCTGTGAATAACAAGACCGAGAATGTCAGCAAACTGGACTACCTTGAGAAG

AS L Y E F L N T S Y I C H K Y I L V S E S T M N E K I L K Q Y 807  
gp190<sup>n</sup> T A A T T A T A T T T G T A T C A A A T A A T 2430  
gp190<sup>s</sup> CTCTATGAGTCTCTGAATACATCTACATCTGCCCAAAATATATCTCTCTCTCACAGCACTATGAACGAGAAGATTCTTAAACAGTAC

AS K I T K E E E S K L S S C D P L D L L F N I Q N N I P V M Y 837  
gp190<sup>n</sup> A T A G A A C T A A G T A T A T A T A A T A T A 2520  
gp190<sup>s</sup> AAGATAACCAAGGAAGAGAGAGTAAGCTGTCTCTGTGTATCCACTGGAGCTGCTGTTCAATATCCAGAACAACTTCCCGTTATGTAT

AS S M F D S L N N S L S Q L F M E I Y E K E M V C N L Y K L K 867  
gp190<sup>n</sup> T T A T A A G T A A A T A T A A A T T T T A T G 2610  
gp190<sup>s</sup> TCTATGTTGAGTACGCTCAACAACTTCTCTCTCAACTGTTCTATGGAGATATATGAGAAGGAGATGCTTGCAGCTGTATAACTCAAA

AS D N D K I K N L L E E A K K V S T S V K T L S S S S M Q P L 897  
gp190<sup>n</sup> T T A A T T A T A G A A A A A A T A A G T T C A A T A 2700  
gp190<sup>s</sup> GACAAGCACAAGATTAAGAAGCTTCTGGAGGAGCTAAGAAGGTCTCCACCTCTGTTAAACTCTCTCTTCCAGCTCCATGCAACCACTG

AS S L T P Q D K P E V S A N D D T S E S T N L N N S L K L F E 927  
gp190<sup>n</sup> A T A G T A A T A T T A A T T A T T G A G T T A T A 2790  
gp190<sup>s</sup> TCTCTCACACCTCAAGACAAGCCGAGTGAAGGCTTAACGACGACCTCTCTCTGACCAACCTTAATAACTCACTGAAACTGTTTGAG

AS N I L S L G K N K N I Y Q E L I G Q K S S E N F Y E K I L K 957  
gp190<sup>n</sup> A T A G T A A C A T A T A A T A A A G T A G T A T T A T A 2880  
gp190<sup>s</sup> AACATCTCTGCTCTCGGCAAGAATAAGAACATCTACCAAGAACTTATTTGGACAGAARTCTGTCGAGAAGTCTACGAGAAGTACTGAAA

AS D S D T F Y N E S F T N F V K S K A D D I N S L N D E S K R 987  
gp190<sup>n</sup> T T T T T A T C T T A T T A T T T A T T A T A G 2970  
gp190<sup>s</sup> GACAGCGACACATTTCTATAACGAGAGCTTCACTAACTTCTGTAATCTAAAGCCGATGATCAACTCTCTTAACGATGAATCTTAACGT

AS K K L E E D I N K L K K T L Q L S F D L Y N K Y K L K L E R 1017  
gp190<sup>n</sup> A T A A T T A T A A A A T T A G T A T C A T T A T T A T A 3060  
gp190<sup>s</sup> AAGAAGCTGGAAGAGGACATCAATAAGCTGAAGAAGACACTGCAACTGAGCTTGCAGCTGTACAACAAGTACAAACTGAAACTGGAGAGA

AS L F D K K K T V G K Y K M Q I K K L T L L K E O L E S K L N 1047  
gp190<sup>n</sup> T A T T A A T T A A A T A A C T T A T A A A A T A T C A T G T 3150  
gp190<sup>s</sup> CTCTTCGACAAGAAGAAGACAGTCCGCAAGTATAAGATGCAAGATCAAGAAGTTGACTCTGCTCAAGGAGCAGCTTGAAGCAAACTCAAC

AS S L N N P K E V L Q N F S V F F N K K K E A E I A E T E N T 1077  
gp190<sup>n</sup> T T C A G T T A A T T T T A A A T A A A T A A A T A 3240  
gp190<sup>s</sup> TCATGTAACATCCGAAACAGTACTGCAGAACTTCTCAGTGTCTCTCAACAGAAGAAGGAAGCCGAGATCCGCGAGACAGAGAACACT

AS L E N T K I L L K H Y K G L V K Y Y N G E S S P L K T L S E 1107  
gp190<sup>n</sup> T A A A A A T A T T A T T A T A A A A T A A T A A G T A 3330  
gp190<sup>s</sup> CTGGAGAACCAAGATTTCTCTCAACACTACAAAGCCCTCGTCAAGTATTATAATGGCGAGTCTTCTCTCTGAAGACTCTCTCCGAG

AS E S I Q T E D N Y A S L E N F K V L S K L E G K L K D N L N 1137  
gp190<sup>n</sup> A T C A T A A A T T T T A A T A A T A A A T A A T T T A T 3420  
gp190<sup>s</sup> GAGAGCATCCAGACCCAGGATAAATACGCCAGCCCTCGAGAAGTCAAGGCTCTGTCTAAGCTCGAAGGCAAGCTGAAGGACAACCTGAAC

09/269874-000299

stop codon Cla 1

Fig. 3D

	N'-terminus	C'-terminus
gp190s1 Sequence		
DNA Sequence	GC <u>ACGCGTATGAA</u> AATC	----- AGCTCTAATTAAATAGGCGGCCGCATCGATGGC
AA Sequence	Mlu I Met Lys Ile	----- Ser Ser Asn stop codon Not I
AA Position	1 2 3	----- 1619 1620 1621
gp190s2 Sequence		
DNA Sequence	GC <u>GGATCCG</u> TGACCCAC	----- AGCTCTAATTAAATAGGCGGCCGCATCGATGGC
AA Sequence	BamH I Val Thr His	----- Ser Ser Asn stop codon Not I
AA Position	20 21 22	----- 1619 1620 1621

Fig. 4A

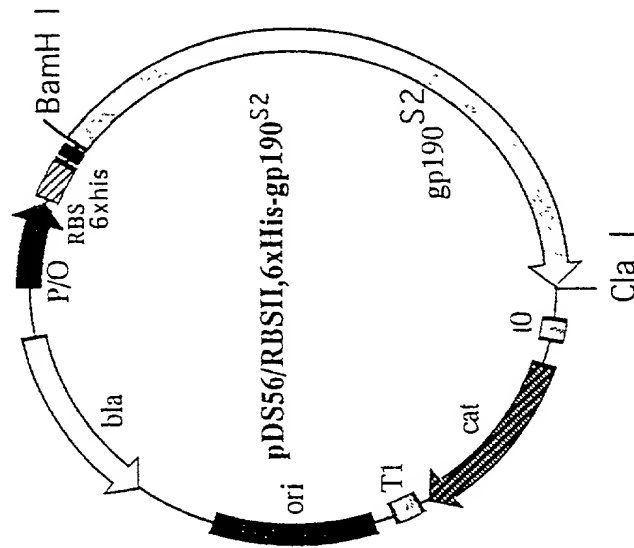


Fig. 4B

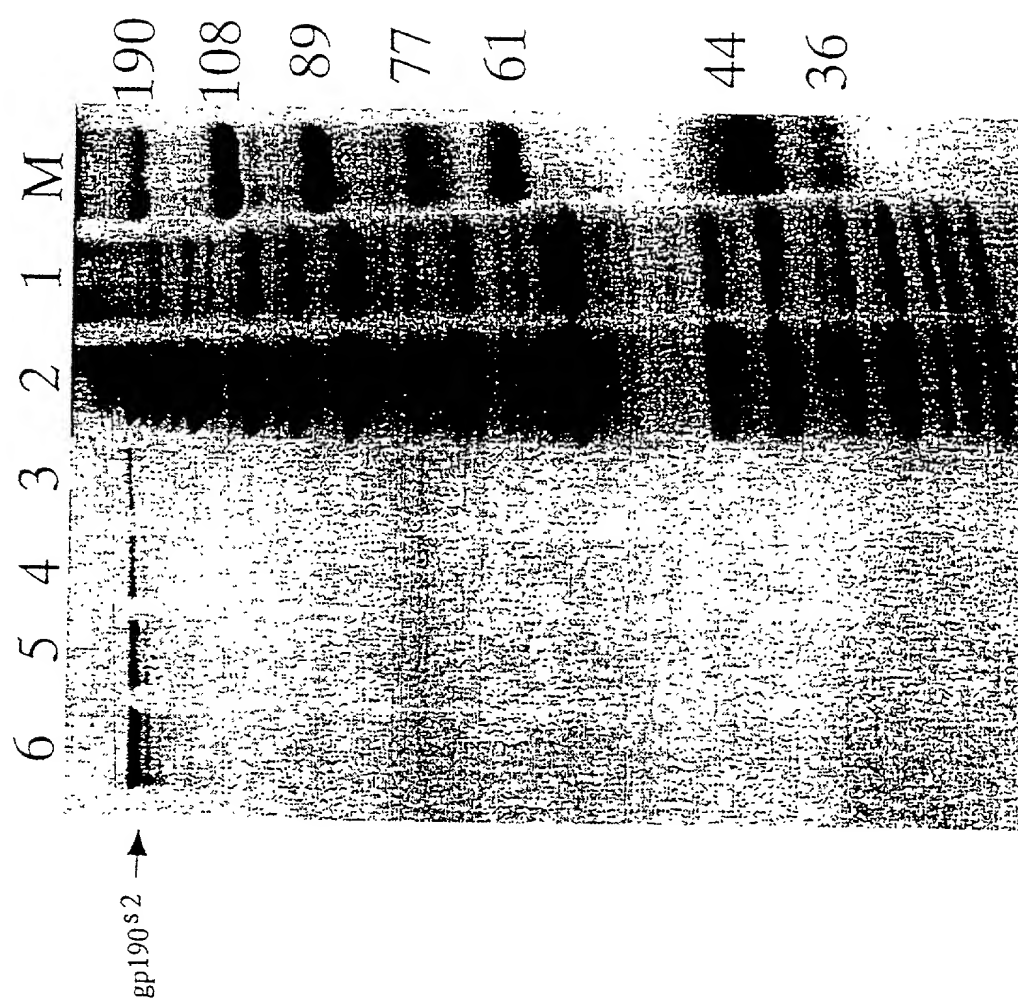


Fig. 5A

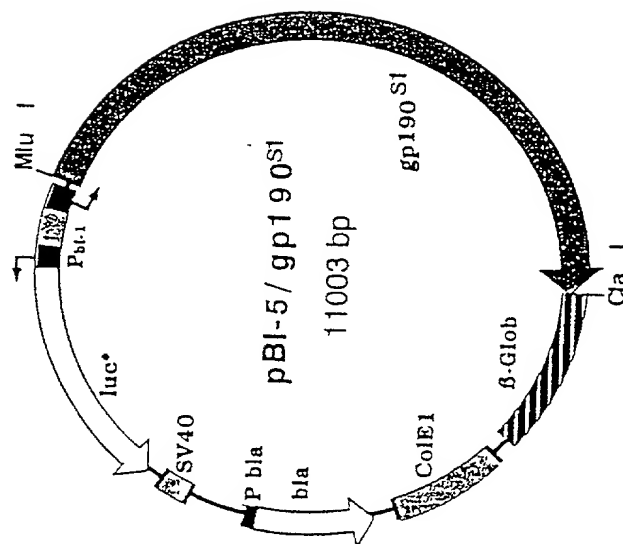


Fig. 5B

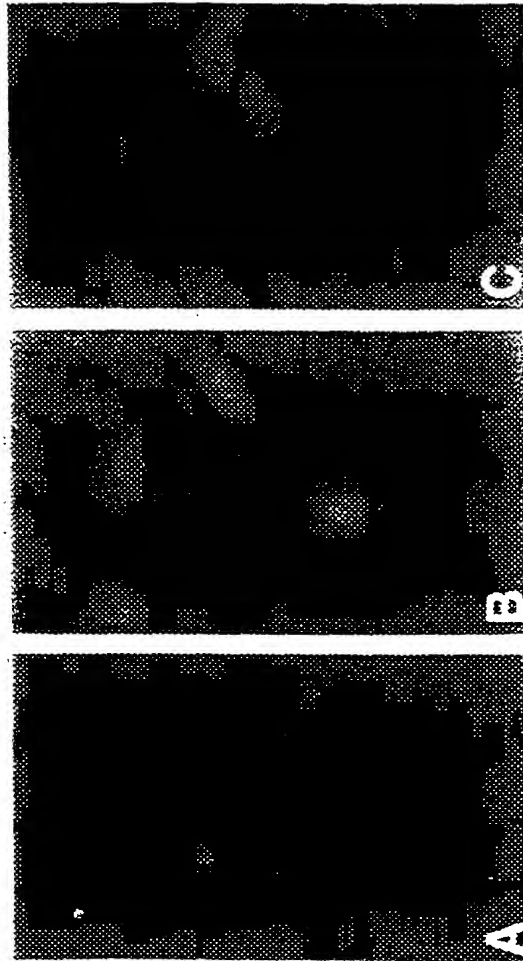


Fig. 5C

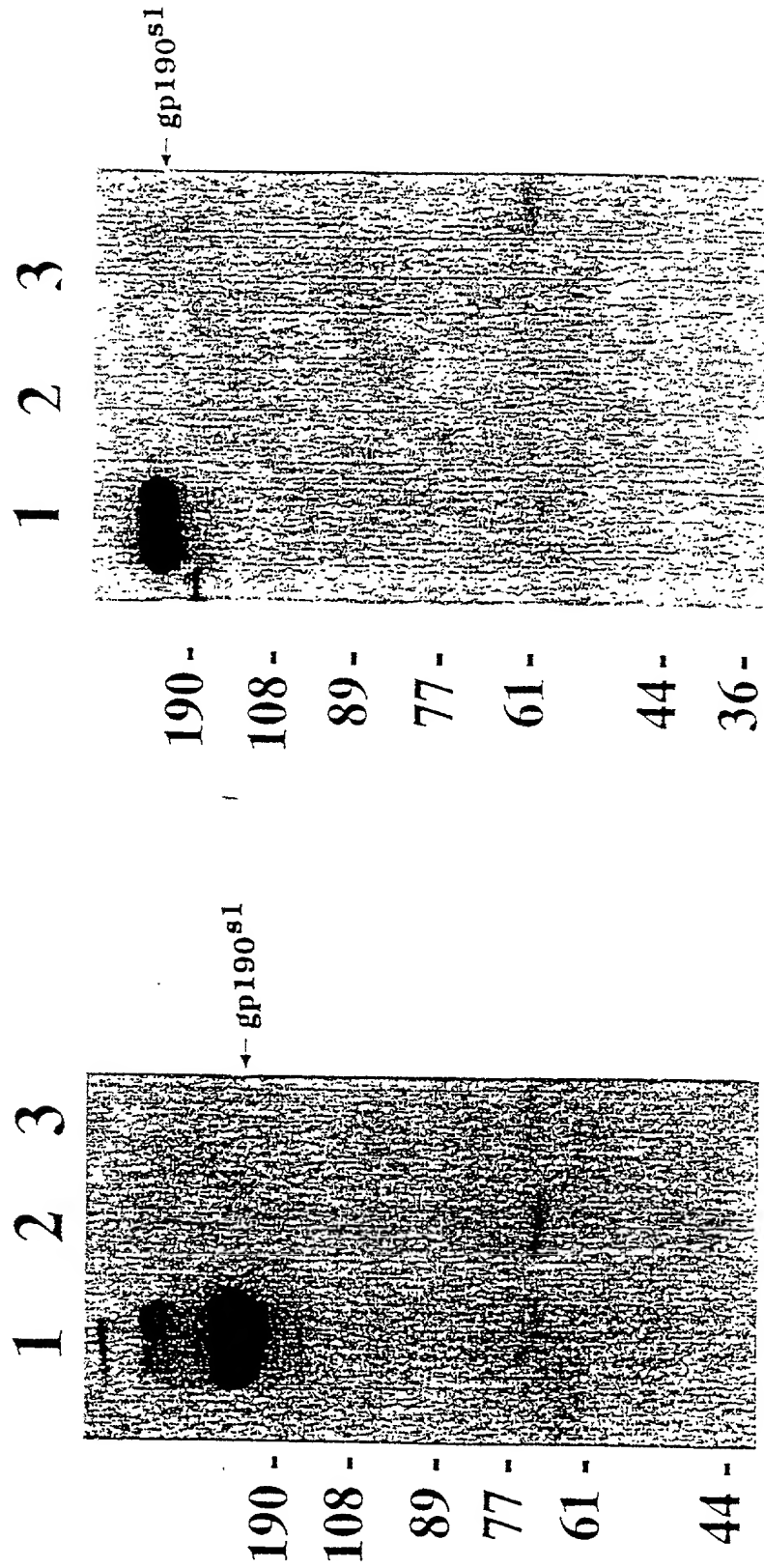




Fig. 6A

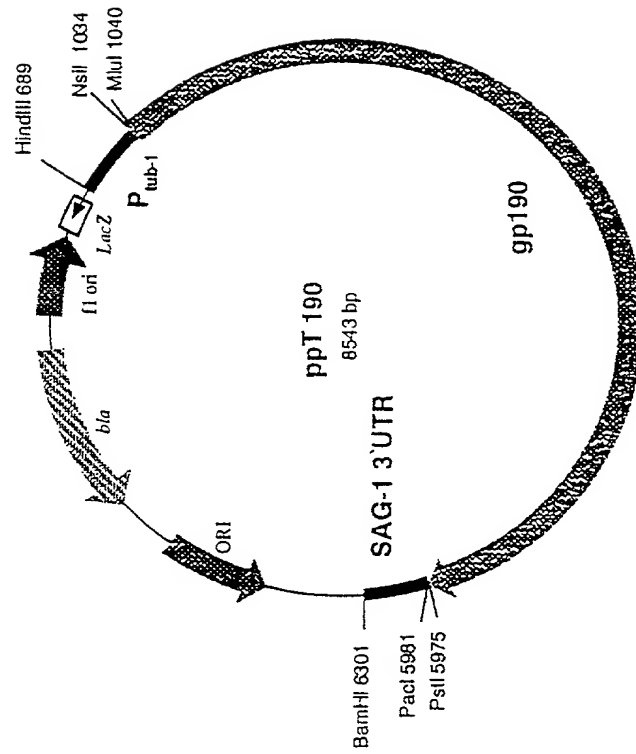
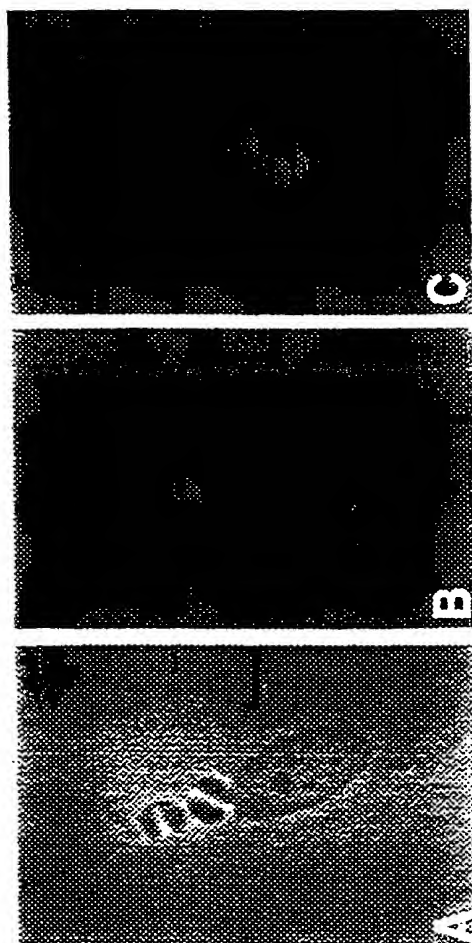


Fig. 6B



0 662080 42869260 0

3

2

1

Fig. 6c

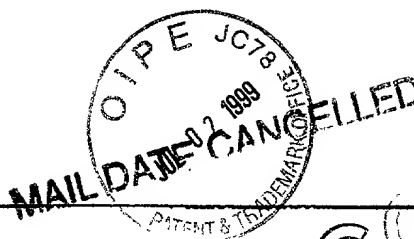
190

108

89

77

61



Patent  
Docket No. 402162000200

**DECLARATION FOR PATENT APPLICATION**  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER  
402162000200

As below named inventors we hereby declare that:

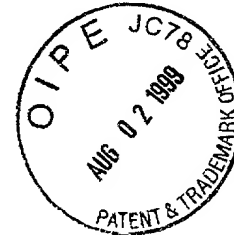
Our residence, post office address and citizenship are as stated below next to our names,

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

RECOMBINANT PROCESS FOR PREPARING A COMPLETE MALARIA ANTIGEN, GP190/MSP1

the specification of which (check only one item below):

- ☐ is attached hereto.
- ☐ was filed as United States application
- Serial No.  
on ,  
and was amended on (if applicable).
- ☒ was filed as PCT international application
- Number PCT/EP97/05441  
on October 2, 1997.



We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37 Code of Federal Regulations § 1.56(a) and (b).

We hereby claim foreign priority benefits under Title 35 United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

**PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119:**

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. § 119
Germany	196 40 817.2	02.10.1996	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

**Declaration for Patent Application (Continued)**  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER  
402162000200

We hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT International application(s) designating the United States of America that are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

**PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120:**

U.S. APPLICATIONS			STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
PCT APPLICATIONS DESIGNATING THE U.S.			STATUS (Check one)		
PCT APPLICATION NUMBER	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)	PATENTED	PENDING	ABANDONED (Expired)

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number)

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201	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY	
202	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY	
203	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY	

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE	DATE	DATE